REMARKS

The specification has been amended merely to introduce a reference to corresponding SEQ ID NO's in the figure legends. No new matter has been added.

Claims 54, 55 and 57 have been amended. New claims 81-85 have been added. The amendments are supported throughout the application as filed, e.g., at page 21, lines 15-27; page 30, lines 11-22. No new matter has been added. Claims 54-82 are pending.

Formal drawings are being submitted herewith.

Rejections Under 35 U.S.C. § 112

Enablement

Claims 54-80 are rejected because "the specification, while being enabling for treating a disorder by administering a cell expressing TSP-2, does not reasonably provide enablement for a functional fragment or analog thereof." The present claims are directed to a method of treating a subject having a disorder characterized by unwanted cell proliferation, including administering to the subject a cell expressing TSP-2 or a functional fragment or analog thereof. According to the Examiner,

> "...it would be an undue burden to one of ordinary skill in the art to assay for claimed sequences, which are capable of functioning as contemplated. One cannot extrapolate the teachings of the specification to the breadth of the claims because the claims are broadly drawn to any functional fragments or analogs of TSP-2, without indicating what function the fragments and/or analogs must possess, and applicant has not enabled all of these types of modifications because it has not been shown that these molecules are capable of functioning as that which is being disclosed."

This rejection has been met by amending claim 54 to delete the "analog" language and reciting that the TSP is at least 95% identical to the sequence of SEQ ID NO:2. In addition, the claims have been amended to require that the TSP-2 or functional fragment thereof be capable of inhibiting endothelial cell migration. Combined with the high level of skill in the art, the specification provides sufficient guidance for a skilled artisan to practice the full scope of the

claimed methods without undue experimentation. In the present case, the TSP-2 sequence is known (SEQ ID NO:2), and molecular biology techniques for making TSP-2 fragments and variants as recited in the claims are routine in the art. In addition, the specification provides guidance for making variants that are highly similar in sequence, as required by the claims, e.g., TSP-2 having conservative amino acid substitutions (see, e.g., pages 54-55). The specification describes the inhibition of tumor growth by TSP-2 in at least 2 different in vivo models: inhibition of squamous cell carcinoma cell grafts (A431 cells) and malignant melanoma cell grafts (MeWo cells) in mice (see, e.g., page 38, line 22 to page 39, line 25, and Figure 3A and 3B).

The specification clearly shows that TSP-2's anti-tumor growth activity correlates with its ability to inhibit endothelial cell migration (see, e.g., page 38, lines 11-21; and page 40, line 21, to page 41, line 19). Further, the specification provides at least 2 assays that can be used to identify TSP-2 fragments that have the required endothelial cell migration inhibitory activity and/or tumor inhibition activity. For example, an *in vitro* human dermal microvascular endothelial cell (HDMEC) migration assay is described in detail at pages 44-45; and a xenograft tumor growth assay combined with a vessel density assay in nude mice is described at page 38, line 27, to page 39, line 25. The specification reports the use of the HDMEC assay to successfully identify active fragments. Indeed, the HDMEC migration assay alone was sufficient to identify a working example of an active TSP-2 fragment from only five fragments that were tested. In particular, applicants found that one of five (20%) of the TSP-2 fragments tested had HDMEC migration inhibitory activity of a level comparable to that of full length TSP-2 (47.6% inhibition by the peptide compared to 54.2% inhibition by full length TSP2) (see page 45). That one of merely five tested fragments gave a positive result is clearly indicative of the routine nature of the assay.

Moreover, we enclose herewith a copy of a declaration of Dr. Michael Detmar under 37 C.F.R. §1.132, originally filed in the parent application. As can be seen from the declaration, the inventors have identified another fragment of TSP-2 that works in the claimed methods, using the techniques described in the application. The inventors made an N-terminal fragment of human TSP-2 (hTSP-2/NTF), encoded by nucleotides 213-1883 of SEQ ID NO:1 (see Figure 1 of declaration), containing the procollagen homology domain and 3 type-1 repeats of TSP-2.

The fragment was effective to inhibit the migration of HDMEC cells as shown by using the same HDMEC assay as described in the specification (see Figure 2 of the declaration). The fragment was also effective to inhibit angiogenesis and growth of squamous cell carcinoma in vivo in mice using the same A431 xenotransplant assay as described in the specification (see Figures 3-5 of the declaration). Thus, the evidence presented in the enclosed declaration of Dr. Detmar clearly shows that one of ordinary skill in the art could identify functional fragments for use in the claimed methods using the guidance provided in the specification.

Further, we have enclosed herewith a copy of an article by Streit et al. (Cancer Res 2002 Apr 1;62(7):2004-12). The article shows that TSP-2 cell therapy can inhibit tumor growth. To wit, the abstract of the article states:

> Fibroblasts were retrovirally transduced to overexpress TSP-2 and were seeded onto biodegradable polymer scaffolds. After transplantation into the peritoneal cavity of nude mice, bioimplants maintained high levels of TSP-2 secretion over extended time periods, resulting in increased levels of circulating TSP-2. Bioimplant-generated TSP-2 potently inhibited tumor growth and angiogenesis of human squamous cell carcinomas, malignant melanomas, and Lewis lung carcinomas that were implanted at a distant site.

Thus, the article clearly shows that one of ordinary skill in the art could perform the claimed methods using the guidance provided in the specification. Accordingly, Applicants respectfully request that the rejection be withdrawn.

Written Description

Claims 54-80 are rejected under 35 U.S.C. 112, first paragraph for written description issues. The Examiner states that the specification

> does not disclose any evidence regarding the treatment of unwanted cell proliferation by administering cells expressing a functional fragment or analog of TSP-2. Likewise, it does not disclose the isolation of and assaying of fragments or analogs of TSP-2 to determine if they possess the same biological activity as TSP-2. In addition, no other examples are disclosed that conveys to one of skill in the art that the applicant was in possession of the claimed analogs or fragments or analogs. There is no actual reduction to practice, sufficient descriptive information, such as definitive structural features, which are critical to polypeptide activity, or complete detailed description of the function of the

> claimed invention indicating that the claimed fragments and analogs were indeed isolated, produced, and assayed for the uses disclosed."

The rejection has been met by amending claim 54 to delete the term "analog" and to recite that the TSP is at least 95% identical to the sequence of SEQ ID NO:2. In addition, the claims, as amended, recite a specific, readily assayable function for the recited TSP molecules. Applicants respectfully traverse the rejection insofar as it may be applied to the presently pending claims.

The pending claims fully satisfy the written description requirement under Federal Circuit law and the Patent Office's own Written Description Guidelines (the Guidelines). The Guidelines state that the written description requirement can be satisfied by:

> sufficient description of a representative number of species by actual reduction to practice. . . .or by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show the applicant was in possession of the claimed genus. (Federal Register, Vol. 66, No. 4, at page 1104).

The Examiner is directed to Example 14 of the Synopsis of Application of Written Description Guidelines (the Synopsis of Application), which indicates that claims reciting a sequence having 95% identity to a disclosed SEQ ID NO and an assayable function are adequately described, even where a single species is disclosed. The claimed TSP-2 polypeptides recited in the claimed methods are limited structurally in that they have 95% identity to SEQ ID NO:2. They are also limited functionally in that they must have the function of inhibiting endothelial cell migration. With regard to claim 81 in particular, the Examiner is directed to Example 9 of the Synopsis of Application of Written Description Guidelines (the Synopsis of Application), which indicates that claims reciting sequences very similar to those of claim 81 are adequately described. The Examiner is reminded that the Federal Circuit, in *Enzo Biochem, Inc. v. Gen-Probe Inc.* (296 F.3d 1316, Fed. Cir. 2002), has taken judicial notice of the Guidelines and the Synopsis of Application. For example, Referring to Example 9 of the Synopsis of Application, the Court stated "[the PTO] has determined that such claims may be adequately described if they hybridize under highly stringent conditions to known sequences because such

conditions dictate that all species within the genus will be structurally similar" (*Enzo*,296 F.3d 1316 at 1327).

Further, numerous fragments that can be used in the claimed methods are described in the specification. For example, the specification provides that a TSP-2 polypeptide used in the claimed methods "includes a domain that includes at least one, two or three type 1 repeat(s)" (see page 22, lines 21-22). In addition, the specification provides as follows:

> For example, the peptide can include a PWAEW sequence (about amino acid residues 386 to 390 of SEQ ID NO:2), or the fragment can include a WSPWAEW sequence (about amino acids 384 to 390 of SEQ ID NO:2), or conservative substitutions of either sequence. (Page 23, lines 3-6)
>
> ***
>
> Construct I expresses selectively the N-terminal procollagen domain of TSP-2 (nucleotides 294-1367), the region with the least homology to TSP-1. Construct 2 expresses, in addition, the type I repeats (nucleotides 294-1883) which contain several biologically active sites including two CSVTCG sequences that mediate binding to the CD36 receptor on endothelial cells. Construct 3 expresses the type I repeats (nucleotides 1383-1883) only. Construct 4 expresses the full-length mature TSP-2 molecule, excluding the signal peptide (nucleotides 294-3755) which is provided in the expression vector. Such recombinant proteins can be used for the generation of monoclonal anti-TSP-2 antibodies, for the establishment of a human TSP-2 ELISA, and **for the systemic treatment of experimental tumors**. (page 33, line 28 to page 34, line 7, emphasis added)

Thus, Applicants have disclosed the use of representative polypeptides in the methods as claimed. Further (and contrary to the Examiner's assertion), Applicants have disclosed the isolation and assaying of the disclosed TSP-2 polypeptide fragments to determine if they possess the same inhibitory activity on endothelial cell migration as TSP-2. (See, e.g., page 45). Therefore, one of ordinary skill in the art would understand that Applicants were in possession of the claimed invention at the time of filing.

In sum, the specification provides ample disclosure of specific, common structural features, as well as functional features, that serve to distinguish the polypeptides used in the claimed methods from those outside the claims. Applicants have disclosed the use of representative polypeptides in the methods as claimed. Thus, the claims meet the standard set by the PTO's own written description guidelines and Synopsis of Application, supported by Federal Circuit law. Accordingly, Applicants respectfully request that the rejection be withdrawn.

Attached is a marked-up version of the changes being made by the current amendment.

Applicant asks that all claims be allowed. Please apply any other charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 19 November 2002

for/ Louis Myers
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

Paragraph beginning at page 31, line 18 has been amended as follows:

Figure 7 is a graph showing the effect of HDMEC migration in the presence of various synthetic TSP-2 derived peptides. Peptides 1, 2, 3 and 4 (P1, P2, P3, P4) (SEQ ID NOs:6-9, respectively) were derived from the procollagen domain of TSP-2; peptide 7 (P7) (SEQ ID NO:10) was derived from the first type 1 repeat of TSP-2.

In the claims:

Claims 54, 55 and 57 have been amended as follows:

54. (Amended) A method of treating a subject having a disorder characterized by unwanted cell proliferation, the method comprising:

identifying a subject having a disorder characterized by unwanted cell proliferation; and

administering to the subject a cell expressing a TSP-2 comprising an amino acid sequence at least 95% identical to the sequence of SEQ ID NO:2, or a functional fragment [or analog] thereof, capable of inhibiting endothelial cell migration.

55. (Amended) The method of claim 54, wherein the cell is a genetically engineered cell modified to cause the expression of the TSP-2 or [a] the functional fragment [or analog] thereof.

57. (Amended) The method of claim 55, wherein the cell comprises an exogenous nucleic aid encoding the TSP-2 or [a] the functional fragment [or analog] thereof.

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Overexpression of Thrombospondin-1 Decreases Angiogenesis and Inhibits the Growth of Human Cutaneous Squamous Cell Carcinomas

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The function of the endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1) in epithelial tumor development has remained controversial. We studied the *in vitro* growth characteristics and the *in vivo* tumor xenograft growth of the human squamous cell carcinoma cell lines A431 and SCC-13, stably transfected to overexpress human TSP-1. Overexpression of TSP-1 inhibited tumor growth of A431 xenotransplants, and completely abolished tumor formation by SCC-13 cells. TSP-1 overexpressing A431 tumors were characterized by extensive areas of necrosis and by decreased tumor vessel number and size. The effects of TSP-1 on tumor cell growth were indirect since tumor cell proliferation rates *in vivo* and *in vitro*, anchorage-dependent and -independent growth *in vitro*, and susceptibility to induction of apoptosis by serum withdrawal were unchanged in TSP-1 overexpressing tumor cells. However, TSP-1 overexpression up-regulated the TSP-1 receptor CD36, leading to enhanced adhesion of A431 cells to TSP-1. These findings establish TSP-1 as a potent inhibitor of angiogenesis and tumor growth in carcinomas of the skin. *(Am J Pathol 1999, 155:441–452)*

To grow beyond minimal size and to metastasize, tumors need to induce the growth of new blood vessels (angiogenesis) providing a lifeline for tumor sustenance and waste disposal.[1] Tumor development is associated with increased release of angiogenesis factors, most prominently of vascular endothelial growth factor (VEGF).[2] Several studies have shown that overexpression of angiogenesis factors in experimental tumors leads to enhanced tumor growth and vascularization, and therapeutic inhibition of VEGF activity has been shown to inhibit tumor growth and metastasis.[3–5]

In contrast, much less is known about the expression and biological role of endogenous inhibitors of angiogenesis during carcinogenesis. Several naturally occurring angiogenesis inhibitors have been identified, including thrombospondin-1 (TSP-1),[6] TSP-2,[7] angiostatin,[8] and endostatin.[9] TSP-1 is a 420-kd homotrimeric matricellular glycoprotein that regulates attachment, proliferation, migration, and differentiation of various cell types (for review, see Ref. 10). TSP-1 inhibits proliferation and migration of vascular endothelial cells *in vitro* and inhibits neovascularization *in vivo*, contributing to the normal quiescence of the vasculature.[11] However, controversial data have been reported regarding the role of TSP-1 in epithelial tumor growth and metastasis. TSP-1 protein expression was shown to be inversely correlated to cellular differentiation in several squamous cell carcinoma (SCC) cell lines,[12] and was shown to induce SCC proliferation, adhesion, migration, and invasion of cells *in vitro*.[13–15] Enhancement of *in vitro* tumor cell invasion by TSP-1 has also been reported for breast, lung, and pancreatic carcinoma cell lines.[16–19] Based on the observation that antisense inhibition of TSP-1 in SCC resulted in suppression of tumor growth *in vivo*,[20] it was suggested that TSP-1 may promote tumor growth.[21] In contrast, other studies reported that TSP-1 expression was inversely correlated with malignant progression in human lung, breast, and bladder carcinoma cell lines.[22,23]

In human skin, TSP-1 is deposited in the basement membrane,[24] contributing to the antiangiogenic barrier that separates the avascular epidermis from the vascularized dermis. Recently, we found that TSP-1 expression was down-regulated in squamous cell carcinomas (SCC) of the skin, richly vascularized malignant tumors derived from epidermal keratinocytes (Detmar M, Velasco P, Tognazzi K, Brown LF, unpublished data). Based on these findings, we studied the biological role of TSP-1 for cutaneous carcinoma growth, using stable tumor cell trans-

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fectants in an intradermal xenograft model. Here, we report that TSP-1 overexpression reduced intradermal tumor growth of A431 epidermoid carcinoma cells and completely inhibited intradermal tumor formation of SCC-13 squamous cell carcinomas, although no direct effect on tumor cell proliferation was detected *in vitro* and *in vivo*. TSP-1 overexpressing A431 tumors were characterized by extensive areas of necrosis and by decreased tumor vessel numbers and sizes. TSP-1 overexpression also up-regulated the TSP-1 receptor CD36, leading to enhanced A431 cell adhesion to TSP-1. These findings establish TSP-1 as a potent inhibitor of angiogenesis and growth of cutaneous squamous cell carcinomas.

Materials and Methods

Cell Culture

The human epidermoid carcinoma cell line A431 was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (all purchased from Gibco BRL).

The human cutaneous squamous carcinoma cell line SCC-13,[25] kindly provided by Dr. James Rheinwald (Harvard Medical School, Boston), was maintained in DMEM/Ham's F-12 medium (3:1) supplemented with 5% FBS, 1% L-glutamine, 50 μg/ml insulin, 0.1 U/ml epidermal growth factor, 18.2 mg/ml adenine, and 0.4 μg/ml hydrocortisone (all purchased from Gibco BRL). Human dermal microvascular endothelial cells (HDMEC) were isolated from neonatal foreskins and cultivated as recently described.[26]

Cell Transfection and Selection

A 3.643-kb human TSP-1 cDNA sequence, comprising the full TSP-1 coding sequence (nucleotides 67–3689 of the human TSP-1 sequence; GenBank accession code X04665), was kindly provided by Dr. Luisa Iruela-Arispe, University of California, Los Angeles. The TSP-1 cDNA was cloned into a pGEM7Z vector (Promega, Madison, WI). The sequence was verified by restriction mapping and by direct sequencing using the Sanger dideoxy method. After restriction digestion with *Sac*I, *Sph*I, and *Pvu*I (Gibco BRL), a 3.6-kb fragment was gel purified, blunted, and ligated into a pcDNA3.1Zeo(-) expression vector (Invitrogen, San Diego, CA) which contains a CMV-enhancer-promoter and a Zeocin selection cassette. Subconfluent cultures were stably transfected either with pcDNA3.1Zeo(-) vector containing the full-length human TSP-1 cDNA or with pcDNA3.1Zeo(-) vector alone using the SuperFect transfection reagent (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were split 1:3 into their full growth medium containing 250 μg/ml Zeocin (Invitrogen) to select transfectants. Stably transfected clones were expanded, and 10 clones were characterized for TSP-1 mRNA and protein expression.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from stable transfectants and from intradermal tumors using the RNeasy kit (Qiagen), according to the manufacturer's instructions. The isolated RNA was subjected to electrophoresis and transferred to Biotrans nylon supported membranes (ICN Pharmaceuticals, Costa Mesa, CA). ^{32}P-radiolabeled cDNA probes were prepared with a random primed synthesis kit (Multiprime; Amersham, Arlington Heights, IL). We used a 4.1-kb TSP-1 cDNA probe and a 300-bp human VEGF cDNA probe which recognizes all known VEGF variants. A 2.0-kb human β-actin cDNA probe purchased from Clontech (Palo Alto, CA) was used as a control for equal RNA loading. Blots were washed at high stringency as described[27] and exposed to X-OMAT MR film (Kodak, Rochester, NY) or a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). mRNA expression was quantitated with a Molecular Dynamics scanning densitometer using the ImageQuant software.

Western Blot Analysis

Western Blot analyses were performed on cell lysates and conditioned media from stably transfected A431 cells, SCC-13, and HDMEC. Cells were grown to confluence in 100-mm dishes, washed with phosphate buffered saline (PBS), and lysed as described.[28] Cell lysates were homogenized using a cell shredder (Qiagen), and protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Conditioned media were obtained from confluent cells grown for 48 hours in serum-free culture medium. TSP-1 was concentrated using heparin beads (Sigma, St. Louis, MO). All samples were boiled in denaturating sample buffer, and equal amounts according to the protein assay were electrophoresed on polyacrylamide gels under reducing conditions.[29] Proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad). To verify equal protein loading membranes were stained with 0.1% Ponceau red (Sigma) diluted in 5% acetic acid. Membranes were incubated overnight in PBS containing 0.1% Tween-20 and 3% bovine serum albumin to block nonspecific binding. Membranes were then incubated with primary antibodies directed against human TSP-1 (clone 133; Genzyme, Cambridge, MA), human CD36 (clone 1A7; NeoMarkers, Fremont, CA), or human VEGF (clone 2352, kindly provided by Dr. Don Senger, Beth Israel Deaconess Medical Center, Boston, MA), washed in PBS/Tween, incubated with horseradish peroxidase-conjugated anti-mouse IgG (Amersham), and analyzed by the enhanced-chemiluminescence system (Amersham). Protein expression was quantitated with a Molecular Dynamics scanning densitometer using the ImageQuant software.

Cell Growth and Apoptosis Assays

To determine whether TSP-1 overexpression influences tumor cell proliferation, we measured anchorage-dependent and -independent cell growth rates. 1×10^5 A431

cells were plated in duplicate into 100-mm culture dishes and total cell numbers per dish were determined after 2, 4, and 6 days using a hemocytometer. Anchorage-independent growth rates were determined by using a soft-agar assay as described.[30] Ten thousand control transfected or TSP-1 transfected A431 cells were transferred into six 30-mm cell culture dishes with a 2-mm grid (Nunc, Naperville, IL). The dishes were incubated at 37°C and 5% CO_2, and colonies were counted after 8 days. The effects of conditioned media from TSP-1 transfectants or from vector transfected controls on endothelial cell proliferation were determined using the BrdU labeling and detection kit (Boehringer, Mannheim, Germany). Human dermal microvascular endothelial cells were grown in 24-well plates in the presence of cell culture medium or conditioned media, supplemented with 10% FBS for 24 hours. HDMEC were also co-incubated with either TSP-1 (25 μg/ml) and/or a TSP-1 neutralizing antibody (50 μg/ml, clone AB-1, Neomarkers) or an isotype-specific control antibody (clone MOPC 21, Sigma). Cell proliferation was assayed according to the manufacturer's instructions. The absorbance was determined at 405 nm using a microtiter plate reader (Titertek, Huntsville, AL). The results represent the mean values ± SD of four dishes per group. Apoptosis induced by serum withdrawal was studied in subconfluent A431 cell clones after 6 days in serum-free medium. The percentage of apoptotic cells was determined as described,[31] using the Fluorescein-FragEL DNA fragmentation kit (Oncogene, Cambridge, MA) according to the manufacturer's instructions, and a Beckton-Dickinson FACS-Scan (Franklin Lanes, NJ).

Cell Adhesion Assay

Twenty-four-well plates were coated with 20 μg/ml human collagen type I (Sigma) or with 20 μg/ml platelet-derived human TSP-1 (Sigma) for 1 hour at 4°C, followed by 100 mg/ml bovine serum albumin (Sigma) to block remaining protein binding sites. A431 cells (1×10^6 cells/ml) in serum-free DMEM medium (Gibco BRL) were mixed with a blocking mouse monoclonal antibody to human CD36 (Neomarkers) or with mouse control IgG (Sigma) at 5 μg/ml. One hundred μl of the cell suspensions were added to each well and were incubated at 37°C for 60 minutes. Unattached cells were removed by three gentle washes with PBS. Attached cells were fixed with 0.1% glutaraldehyde (Sigma), stained with a 0.1% crystal violet solution (Sigma), and washed three times with PBS. Crystal violet adsorbed onto cells was solubilized with 0.2% Triton X-100 (Sigma), and absorbance was measured at 590 nm in a Titertek microtiter plate reader. The absorbance of 1×10^5 fixed cells served as control (100% value).[32] The unpaired Student's *t*-test was used for statistical analysis of the results.

Growth of Xenografts in Nude Mice

Confluent A431 or SCC-13 cells, stably transfected with a human TSP-1 expression vector or with the expression vector alone, were trypsinized and resuspended in serum-free DMEM medium (Gibco BRL) at a density of 2×10^7 cells/ml. Two million tumor cells of each type were injected intradermally into both flanks of five 8-week-old female BALB/c (nu/nu) mice. Two control clones and 3 TSP-1 overexpressing clones were investigated. The smallest and largest tumor diameter were measured weekly, using a digital caliper, and tumor volumes were calculated using the following formula:

$$\text{Volume} = 4/3 \times \pi \times (1/2 \text{ smaller diameter})^2 \times 1/2 \text{ larger diameter}$$

Mice were sacrificed after 4 weeks in the group of A431 injected animals and after 7 weeks in the group of SCC-13 injected animals or earlier if the largest tumor diameter reached 20 mm. All animal studies were approved by the subcommittee on Research Animal Care of the Massachusetts General Hospital.

In Situ *Hybridization and Immunohistochemistry*

In situ hybridization was performed on 6-μm paraffin sections of tumor xenografts as described.[27] The sense and antisense single-stranded RNA probes for human VEGF were transcribed from a pGEM-3Zf(+) vector containing a 204-bp polymerase chain reaction fragment common to all known VEGF splicing variants. A RNA probe to human TSP-1 was transcribed from a pBluescript II KS+ vector containing a 240-bp polymerase chain reaction fragment of the coding region of human TSP-1. Immunohistochemical staining was performed on 6-μm frozen or paraffin sections of tumor xenotransplants as previously described,[33] using monoclonal antibodies against mouse CD31 (Pharmingen, San Diego, CA), human TSP-1 (Genzyme), human CD36 (Neomarkers), and human PCNA antigen (Zymed Laboratories, San Francisco, CA).

Computer-Assisted Morphometric Analysis of Tumor Vessels

To determine the degree of tumor-induced angiogenesis, cryostat sections of tumor xenografts were stained with an anti-mouse CD31 monoclonal antibody. Representative sections obtained from five tumors from each cell clone were analyzed, using a Nikon E-600 microscope (Nikon, Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP LAB software program (Scanalytics Inc.; Fairfax, VA). Three different fields at 60x magnification were examined on each section, and the number of vessels per mm^2, the size distribution, the average, and the total area of tumor blood vessels were determined. The unpaired *t*-test was used to analyze differences in the vessel areas.

Results

Overexpression of TSP-1 in Two Human Cutaneous Tumor Cell Lines

The human squamous cell carcinoma cell lines A431 and SCC-13 were chosen with respect to their endogenous TSP-1 and VEGF secretion and their *in vivo* growth characteristics. A431 cells are characterized by strong secretion of VEGF but little or no TSP-1 secretion (Figure 1), and form fast growing and highly vascularized tumors *in vivo*.[34] SCC-13 cells express low levels of VEGF but synthesize substantial amounts of TSP-1 (Figure 1), and grow slowly as well differentiated, rounded tumors. The TSP-1 expression levels of multiple TSP-1 and control transfected clones were determined by Northern Blot analyses (Figure 1A). The highest levels of TSP-1 mRNA were detected in A431 clones 10, 12, and 19 and SCC-13 clones 1, 2, and 3. Western blot analyses confirmed that increased TSP-1 mRNA levels correlated with increased amounts of TSP-1 protein. In TSP-1 transfected A431 cell clones, strong expression of the 180 kd TSP-1 protein was found in cell lysates and in culture supernatants, confirming efficient secretion of TSP-1 (Figure 1B). In contrast, little or no TSP-1 protein was detected in A431 cells transfected with vector only. In SCC-13 cell clones, TSP-1 was already expressed by control transfectants (Figure 1B); three TSP-1 transfected clones showed markedly increased TSP-1 secretion although the amount of cell-associated TSP-1 was not significantly increased. TSP-1 expression was also detected in cell lysates but not in conditioned medium harvested from human dermal microvascular endothelial cells (Figure 1B). The biological activity of transfected human TSP-1 was confirmed in HDMEC proliferation assays. HDMEC proliferation was significantly inhibited after a 24 hour incubation with conditioned media harvested from TSP-1 overexpressing A431 and SCC-13 cell clones, as compared to HDMEC incubated with unconditioned media (Figure 1, C and E). Supernatants obtained from control transfected A431 clones stimulated HDMEC proliferation. Incubation with a TSP-1 neutralizing antibody but not with an isotype-control antibody abolished the inhibition of HDMEC proliferation by culture medium supplemented with TSP-1 and by conditioned medium obtained from TSP-1 overexpressing cell clones (1D).

Growth of TSP-1 Overexpressing Tumor Cells in Vitro and Tumor Growth in Vivo

Our results showed no differences in anchorage-dependent cell proliferation between TSP-1 overexpressing A431 clones and control clones growing in plastic culture dishes (Figure 2A). Anchorage-independent cell growth was studied by determination of colony numbers in a soft agar assay. No significant differences in the number of colonies were observed between the different cell clones (Figure 2B). To compare the susceptibility to induction of apoptosis by serum withdrawal, A431 clones were cultured under serum-free conditions for 6 days. No significant differences in the percentage of apoptotic cells were found between TSP-1 overexpressing A431 clones (13.67% ± 4.04%) and control A431 clones (12.33% ± 1.45%). To determine the biological effects of TSP-1 overexpression on the orthotopic tumor growth of A431 and SCC-13 cells *in vivo*, tumor cells were injected intradermally into the flanks of immunodeficient nude mice. Control transfected A431 cell clones formed rapidly growing tumors (Figure 2E), reaching a volume of 2000–3000 mm^3 after 4 weeks. Stable overexpression of TSP-1 resulted in a significant inhibition of tumor growth by 50 to 75% after 4 weeks, as compared to control tumors (Figure 2E). Northern Blot analysis of RNA extracted from two representative tumors of each A431 cell clone confirmed that TSP-1 transfected tumor cell clones maintained TSP-1 mRNA expression *in vivo* (data not shown), with the highest TSP-1 mRNA expression and the slowest tumor growth in A431 clone T10. Northern analysis of the same RNA samples demonstrated equal levels of VEGF mRNA expression in TSP-1 overexpressing and control tumors. The effects of transfected TSP-1 on *in vivo* tumor growth were confirmed in transfected SCC-13 cell clones. After 2 weeks, control transfected SCC-13 clones formed slowly growing intradermal tumors (Figure 2F). In contrast, TSP-1 overexpression in SCC-13 cells led to a complete inhibition of *in vivo* tumor growth in all clones tested (clones T2 and T3) over an observation period of up to 12 weeks (Figure 2F).

Histological Characteristics of TSP-1 Overexpressing Tumors

Extensive areas of necrosis were detected in TSP-1 overexpressing tumors (see Figure 4B), whereas only occasional small necrotic foci were found in control tumors (see Figure 4A). However, the fraction of proliferating cells within the viable tumor areas, as determined by staining for the PCNA antigen, was unchanged (data not shown). The expression and distribution of TSP-1 within A431 tumors was assessed by *in situ* hybridization and by immunohistochemistry. Only weak TSP-1 mRNA expression was detected in control tumor cells (Figure 3, A and B), and TSP-1 protein expression was predominantly found in the dermal-epidermal basement membrane zone of adjacent normal skin and in blood vessels, but not in tumor cells (see Figure 5A). In contrast, strong TSP-1 mRNA expression was detected in TSP-1 overexpressing tumor cells (Figure 3, C and D), and immunohistochemistry demonstrated massive TSP-1 deposits in the tumor stroma (see Figure 5B). In accordance with the Northern Blot results, no major differences of VEGF mRNA expression were found between TSP-1 overexpressing and control tumors by *in situ* hybridization (Figure 3, E–H). Up-regulation of VEGF mRNA expression was observed adjacent to areas of necrosis in tissue samples derived from TSP-1 overexpressing tumors and control tumors.

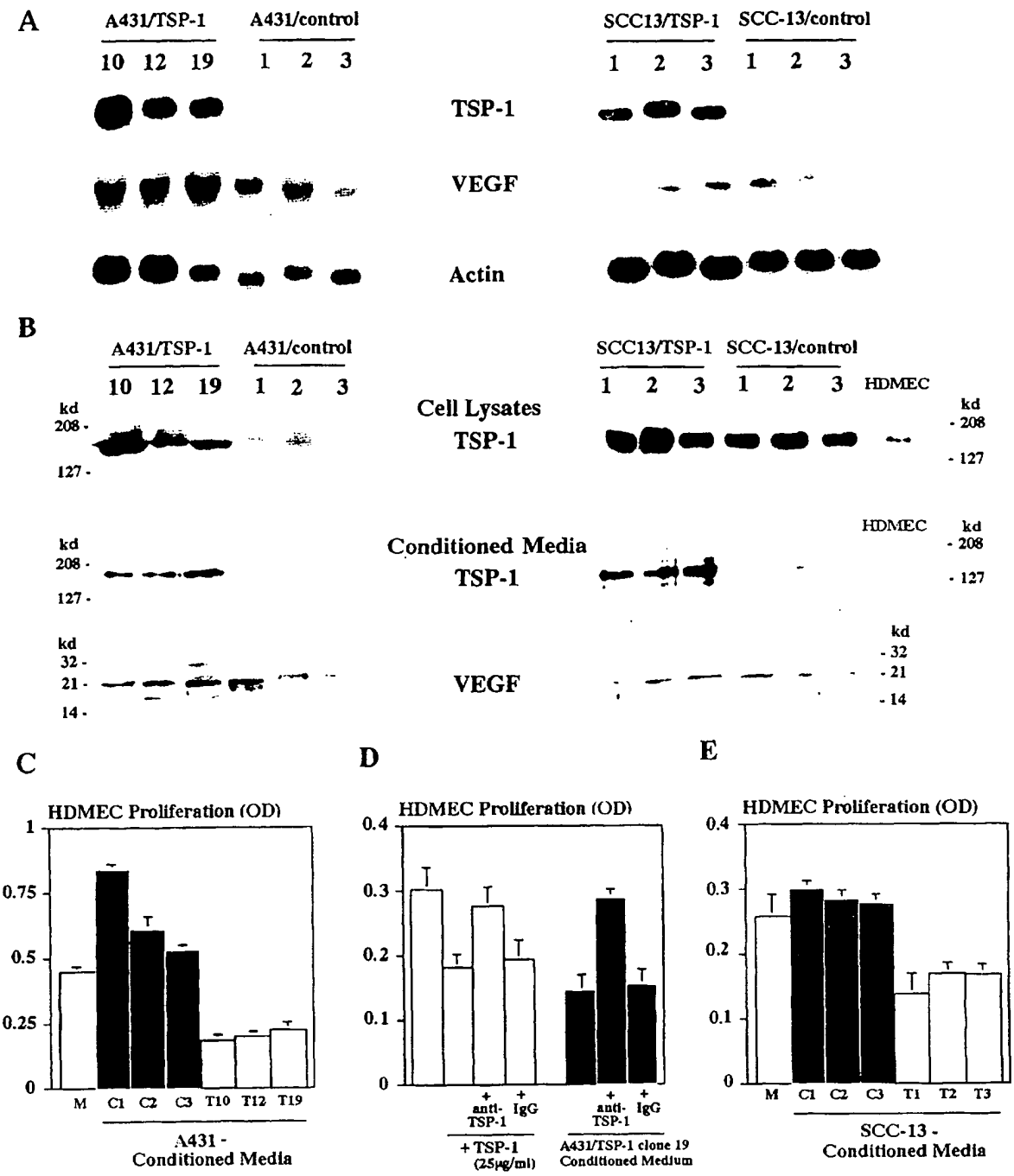


Figure 1. A: Overexpression of human TSP-1 mRNA in stably transfected A431 and SCC-13 cell clones as compared to vector transfected control clones was confirmed by Northern blot analysis. mRNA expression of human VEGF remained unchanged in TSP-1 overexpressing and control clones. Hybridization with a human β-actin probe served as control for equal loading. B: Western blot analysis demonstrated increased amounts of TSP-1 protein in cell lysates and conditioned media obtained from TSP-1 transfected A431 cells. TSP-1 migrates at approximately 180 kd. Control transfected SCC-13 cell clones synthesized substantial amounts of TSP-1. Increased TSP-1 secretion was detected in all three TSP-1 transfected cell clones (C1–3). The secretion of VEGF is not modified by TSP-1 overexpression in A431 and SCC-13 cell clones. HDMEC synthesize but do not secrete detectable amounts of TSP-1. C: Mitogenic effect of conditioned media obtained from control-transfected A431 clones (C1–3) as compared to unconditioned medium (M). Conditioned media obtained from TSP-1 transfected A431 clones (T10, T12, T19) significantly inhibited HDMEC proliferation, as determined by BrdU incorporation ($P < 0.01$). Bars represent mean values ± SD of two independent experiments. D: Co-incubation with a TSP-1 neutralizing antibody (anti-TSP-1) but not with an isotype-specific control antibody (IgG) abolished the inhibitory effect of TSP-1 (25 μg/ml) added to unconditioned medium (open bars) or of conditioned medium harvested from a TSP-1 overexpressing A431 cell clone (T19) (closed bars) on HDMEC proliferation. E: Conditioned media obtained from TSP-1 transfected SCC-13 cell clones (T1–3) but not from control-transfected clones (C1–3) inhibited HDMEC proliferation.

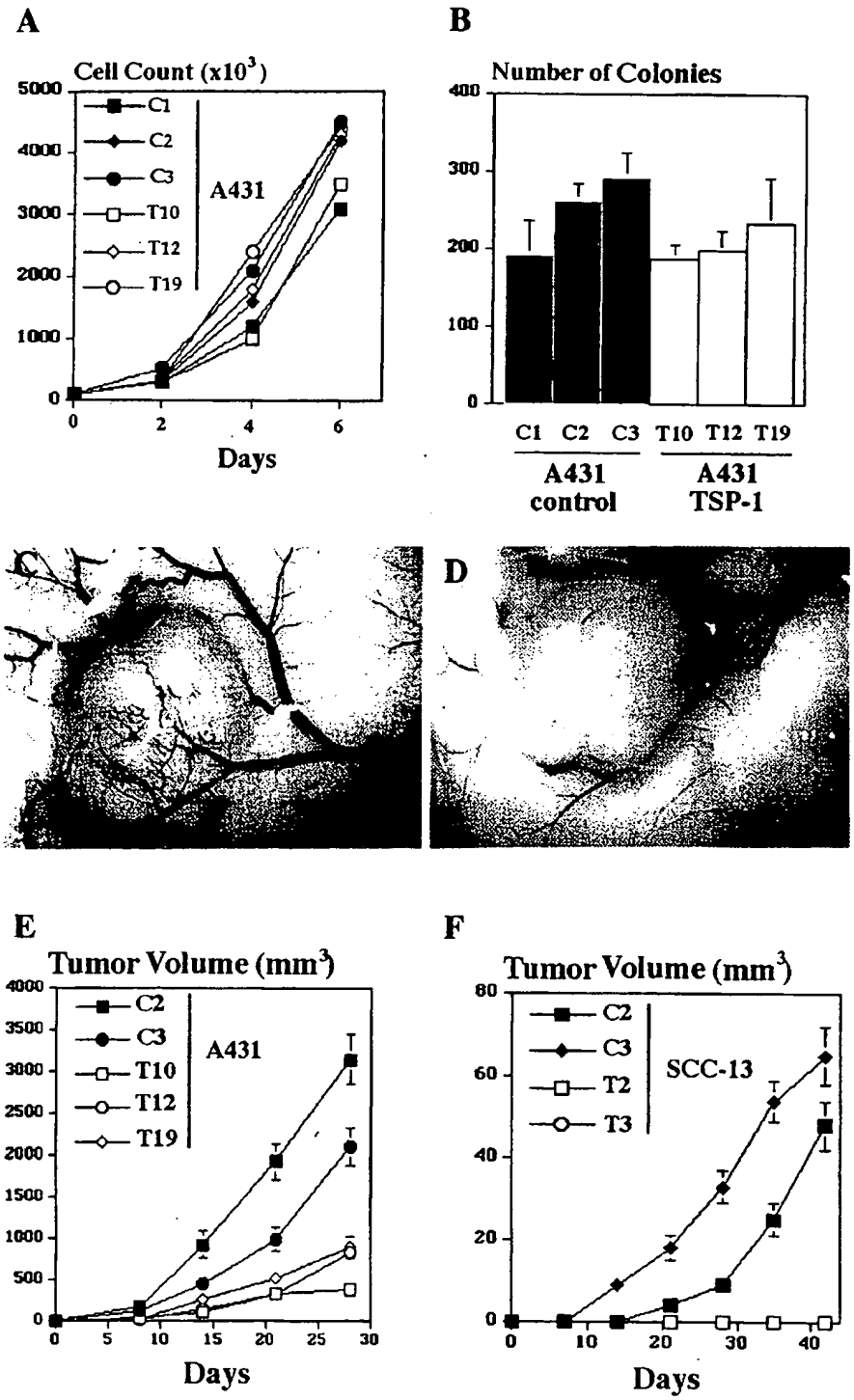


Figure 2. A: No influence of transfected TSP-1 on the anchorage-dependent growth of A431 cell clones (T10, T12, T19), as compared to control transfectants (C1–3). B: No major differences in anchorage-independent growth of TSP-1 transfected and control-transfected A431 clones, as determined in a soft agar assay. Mean values ± SD of two independent experiments. C, D: Rarefication of large tumor blood vessels supplying TSP-1 overexpressing A431 xenotransplants (D), as compared to control transfected A431 tumors (C). Scale bar = 1 cm. E: Decreased tumor growth of TSP-1 overexpressing A431 cells (T10, T12, T19), as compared to control-transfected clones (C2, C3). Values represent mean values ± SEM for 10 tumors for each clone and time point. F: Complete inhibition of tumor growth of TSP-1 overexpressing SCC-13 clones T2 and T3 (values identical to T2), as compared to control clones C2 and C3. Note different scale of the *y* axis, as compared to Figure 2E.

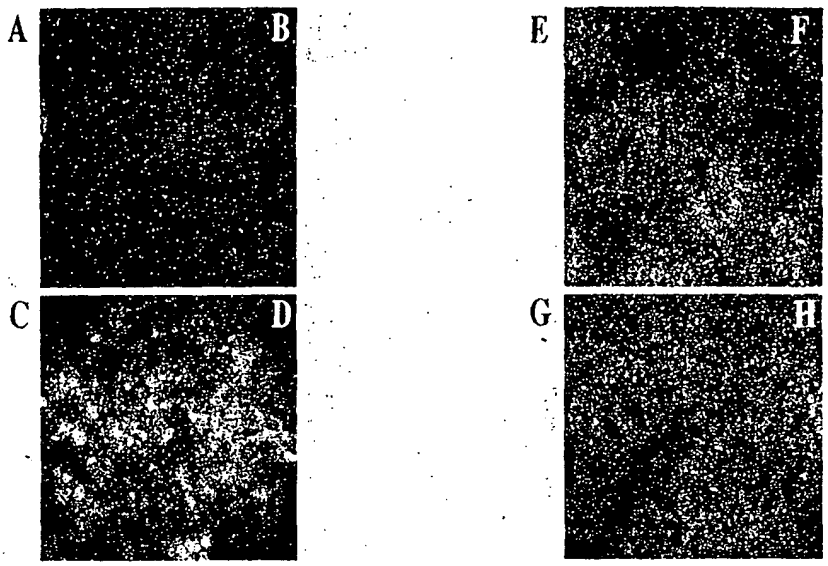


Figure 3. *In situ* hybridization demonstrates strong TSP-1 mRNA expression in 3-week-old TSP-1 transfected A431 xenografts (C, D), whereas little or no TSP-1 mRNA expression was detected in control tumors (A, B). In contrast, VEGF mRNA expression remained unchanged in TSP-1 transfected A431 xenotransplants (G, H), as compared to control tumors (E, F). Scale bar = 200 μm.

Morphometric Analysis of Tumor Vessels in TSP-1 Overexpressing Tumor Xenografts

The morphology of tumor supplying blood vessels was studied on the surface of tumors of identical size derived from TSP-1 transfected A431 clones after 4 weeks and control transfectants after 3 weeks (Figure 2, C and D). A significant rarefaction of large blood vessels was seen selectively on the surface of TSP-1 overexpressing tumor xenografts. To determine the microvascular density within TSP-1 transfected and control A431 tumors, frozen sections of 5 different tumors derived from the same A431 clone were stained with an antibody against mouse CD31 (Figure 4, C and D). Morphometric analysis revealed decreased microvessel densities (Figure 4E) in 3-week-old tumors derived from TSP-1 overexpressing clones T10 (28 ± 10 vessels/mm^2), T12 (46 ± 12 vessels/mm^2), and T19 (32 ± 7 vessels/mm^2), as compared to control clone C2 (60 ± 19 vessels/mm^2) and C3 (52 ± 20 vessels/mm^2). Moreover, the average area per vessel was significantly ($P < 0.001$) smaller in TSP-1 overexpressing tumors (Figure 4G) derived from clone T10 (729 ± 213 μm^2), T12 (551 ± 157 μm^2), and T19 (755 ± 189 μm^2), as compared to control tumors derived from clone C2 (1057 ± 307 μm^2) and C3 (1298 ± 504 μm^2). The fraction of large blood vessels with an area of more than 2000 μm^2 was decreased to fewer than 1% of all vessels in TSP-1 overexpressing tumors, as compared to more than 10% in control tumors (Figure 4H). In accordance with these data, the total area covered by blood vessels (Figure 4F) was significantly ($P < 0.001$) smaller in tumors derived from TSP-1 transfected clones T10 (1.7 ± 0.6%), T12 (2.6 ± 1.2%) and T19 (2.2 ± 0.5%), as compared to control transfected clones C2 (5.8 ± 2%) and C3 (5.4 ± 1.7%).

Up-Regulation of the TSP-1 Receptor CD36 in TSP-1 Transfected Tumor Cells and Xenografts

It has been suggested that the antiangiogenic effect of TSP-1 is mediated through the CD36 receptor on endothelial cells.[35] We studied the expression of CD36 in TSP-1 overexpressing A431 tumors and controls by immunohistological staining. While CD36 expression was nearly absent in control transfected tumors (Figure 5C), strong CD36 expression was found in a substantial number of TSP-1 overexpressing A431 tumor cells (Figure 5D). These findings were confirmed by Western blot analysis, demonstrating strong up-regulation of CD36 in cell lysates obtained from TSP-1 overexpressing A431 (Figure 5E) and SCC-13 (Figure 5F) clones as compared to control clones. When subconfluent A431 control cells (clones 1, 2, and 3) were incubated for 24 hours with conditioned media taken from TSP-1 overexpressing A431 clone T19, a 1.5 to ninefold induction of CD36 expression was detected by Western blot, as compared to incubation with conditioned media from control clones (Figure 5G). In contrast, CD36 was already strongly expressed in untreated HDMEC, and incubation with TSP-1 containing conditioned media did not further increase CD36 protein expression levels (data not shown). To study the biological significance of increased CD36 expression on TSP-1 transfected A431 clones, we studied cell adhesion to immobilized TSP-1 and collagen type I. TSP-1 overexpressing A431 cells showed significantly enhanced cell adhesion onto a TSP-1 matrix ($P < 0.001$) but not onto a collagen type I matrix (Figure 5, H and I). Incubation with a blocking antibody against the CD36 receptor significantly reduced adhesion of TSP-1 overexpressing A431 cells to TSP-1 ($P < 0.001$), but not to collagen type I (Figure 5, H and I). In contrast, the adhe-

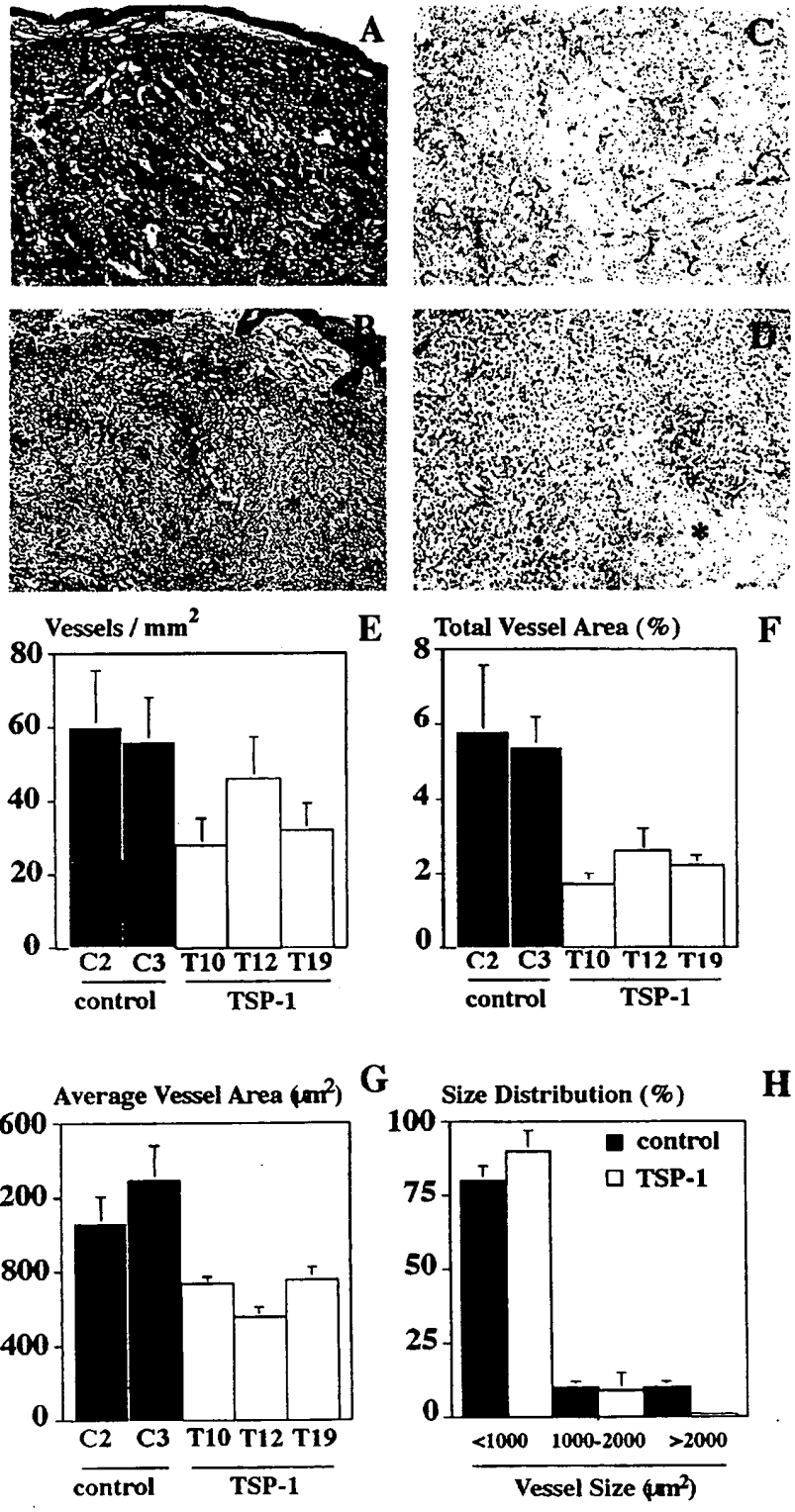
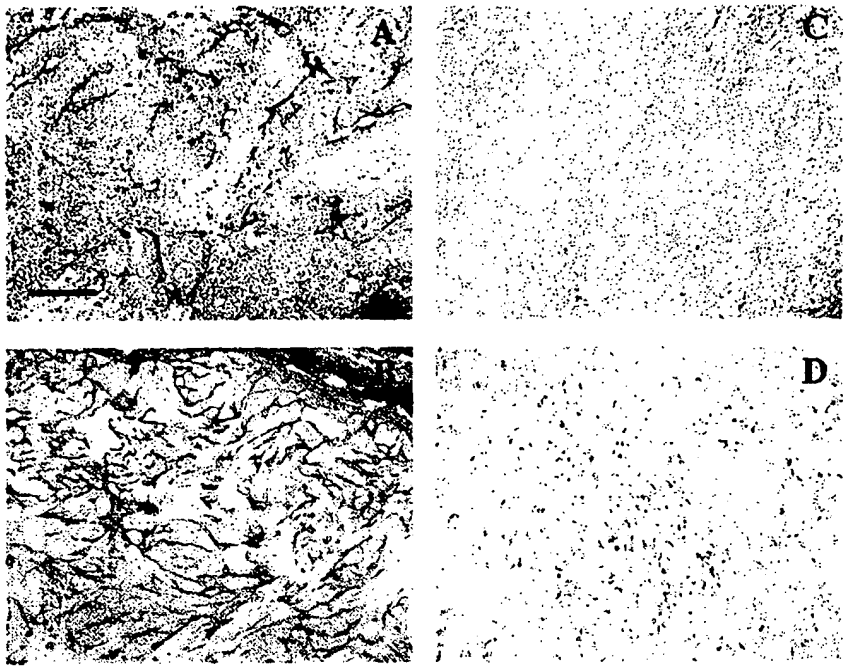
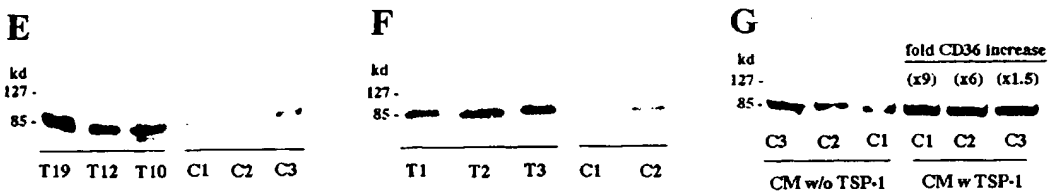


Figure 4. Extensive areas of tumor necrosis (*) in TSP-1 overexpressing A431 xenotransplants (B), as compared to control tumors (A); hematoxylin-eosin stain. Scale bar = 200 µm. Immunohistochemical staining with an anti-mouse CD31 monoclonal antibody revealed inhibition of angiogenesis in TSP-1 overexpressing tumors (D), as compared to control tumors (C). E: Moderate reduction of microvascular densities in TSP-1 overexpressing tumors, as measured by the vessel number per mm² tumor area. F: Significant decrease of the area covered by blood vessels in TSP-1 overexpressing tumors compared to control tumors ($P < 0.001$). G, significant reduction of the average blood vessel area ($P < 0.001$); H, loss of blood vessels of more than 2000 µm² in TSP-1 overexpressing tumors, as compared to control tumors. CD31 stained blood vessels were evaluated in three different 10× fields in sections of five different tumors derived from each A431 cell clone. Data are expressed as mean values ± SEM.





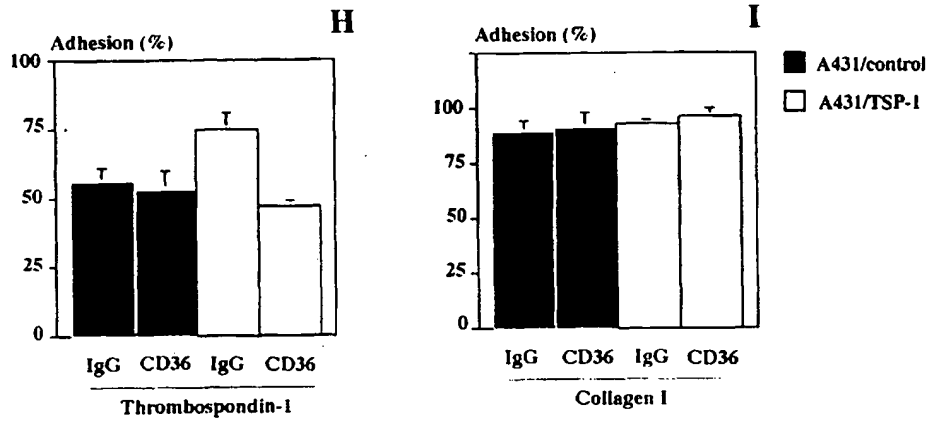


Figure 5. A–D: Immunohistochemical analysis demonstrates strong TSP-1 staining in TSP-1 transfected A431 xenotransplants, predominantly localized in the tumor stroma (B), as compared to the sparse labeling observed in control tumors (A). Little or no detectable CD36 expression in control A431 tumors (C), but strong CD36 expression in TSP-1 transfected xenotransplants (D). Scale bar = 200 μm. E, F: Western blot analysis demonstrates increased CD36 expression in cell culture lysates obtained from TSP-1 transfected A431 clones (T10, T12, and T19) (E) and SCC-13 cell clones (T1-T3) (F), as compared to control clones. G: CD36 expression is induced in cultured A431 cells (C1, ninefold; C2, sixfold; C3, 1.5-fold) after 24 h incubation with conditioned medium obtained from TSP-1 overexpressing clones. H: Significantly increased adhesion of TSP-1 transfected A431 cells to immobilized TSP-1, as compared to control cells ($P < 0.001$). Incubation with an anti-CD36 blocking antibody significantly ($P < 0.001$) reduced adhesion of TSP-1 transfected A431 cells to levels observed in control cells. I: No significant difference in adhesion to collagen type I between control and TSP-1 transfected A431 cells. An anti-CD36 blocking antibody did not modify A431 cell adhesion to collagen type I. Scale bars represent mean values ± SD of two independent experiments.

sion of A431 control cells to TSP-1 or collagen type I was not affected. These results suggest that overexpression of TSP-1 in A431 and SCC-13 tumor cells leads to enhanced expression of its receptor CD36 with potential consequences for tumor cell adhesion.

Discussion

Conflicting results have been reported regarding the function of TSP-1 in tumor progression. We chose two established epithelial skin cancer models to enhance tumor cell TSP-1 expression by stable transfection. Our study was designed to characterize the effects of transfected TSP-1 on the orthotopic tumor growth of experimental skin cancer and to characterize the mechanisms by which TSP-1 exerts these effects.

Overexpression of TSP-1 in A431 cell xenotransplants potently decreased tumor growth, as compared to control tumors transfected with vector only. Increased TSP-1 secretion by stable transfectants was confirmed by Western blot analyses of conditioned media, and the bioactivity of transfected TSP-1 was confirmed in a proliferation assay using human dermal microvascular endothelial cells which by themselves did not secrete substantial amounts of TSP-1. Addition of conditioned media from TSP-1 transfected A431 clones significantly inhibited HDMEC proliferation, whereas conditioned media from control A431 clones stimulated HDMEC growth. The potent inhibitory effect of conditioned media from TSP-1 transfected cell clones on HDMEC proliferation was TSP-1 specific since it was abolished by co-incubation with a TSP-1-neutralizing antibody. *In situ* hybridizations of tumor xenotransplants demonstrated that TSP-1 mRNA expression was maintained at high levels in TSP-1 transfected tumor cell clones. Together, these data provide evidence for a potent inhibitory effect of TSP-1 on skin cancer growth. In accordance with our results, TSP-1 has been reported to inhibit the *in vivo* growth of MDA-MB-435 breast carcinoma cells[36] and of v-*src* transformed NIH 3T3 fibroblasts.[37] Furthermore, TSP-1 negatively affected the tumor forming ability of transformed mouse endothelial cells,[38] of human glioblastoma cells,[39] and of human B16/F10 melanoma cells.[40] However, a previous investigation using the human squamous cell carcinoma cell line 11B reported that reduction of TSP-1 secretion by antisense transfection inhibited tumor growth *in vivo*, and the authors suggested that TSP-1 overexpression in squamous cell carcinomas might lead to enhanced tumor growth.[20] Our data provide strong evidence against this hypothesis and demonstrate, furthermore, that TSP-1 may even completely prevent the formation of cutaneous squamous cell carcinomas. We could demonstrate that stable TSP-1 transfection of human SCC-13 cells, a slowly growing human squamous cell carcinoma line with formation of highly differentiated tumors *in vivo*, induced a complete inhibition of any detectable tumor growth over an observation period of 12 weeks after xenotransplantation.

The tumor growth inhibition induced by TSP-1 in cutaneous squamous cell carcinomas was not due to direct TSP-1-mediated inhibition of tumor cell growth. We used three independent *in vitro* assays to investigate TSP-1 effects on A431 cells: (1) Anchorage-dependent cell growth in monolayer culture; (2) anchorage-independent cell growth, as determined by the ability to form colonies in soft agar; and (3) susceptibility to induction of apoptosis by serum withdrawal. No significant differences between TSP-1 transfected A431 clones and control transfected A431 clones were detected in any of these studies. These findings are in accordance with previous findings, showing that TSP-1 transfection did not influence the *in vitro* growth of human MDA-MB-453 breast cancer cells[36] and B16/F10 melanoma cells.[40] However, they are in contrast to a previous investigation, reporting a direct correlation between the levels of TSP-1 secretion by several squamous cell carcinoma cell lines and enhanced *in vitro* cell growth and invasion.[41] Moreover, another study reported that antisense-mediated downregulation of TSP-1 expression in a squamous cell carcinoma cell line led to decreased *in vitro* cell proliferation, suggesting TSP-1 as a promoter of tumor growth.[20] In 3T3 fibroblasts, antibodies to TSP-1 were reported to inhibit cell proliferation, and TSP-1 overexpression induced serum- and anchorage-independent growth.[42] While there is no obvious explanation for these discrepancies, varying susceptibilities of different cell types (eg, mesenchymal *versus* epithelial) or of distinct cell lines to TSP-1 may play a role. It has been reported that TSP-1 inhibited proliferation of A2058 malignant melanoma cells *in vitro*,[43] and certain TSP-1 effects seem to be specific for distinct cell lines. Our results, obtained by using three independent *in vitro* growth assays, strongly indicate that A431 cell growth is not influenced by TSP-1. This is further supported by the findings that cell proliferation rates of TSP-1 overexpressing A431 xenotransplants were indistinguishable from control transfected A431 xenotransplants, as measured by expression of the proliferation-related antigen PCNA. Similarly, tumor treatment with the angiogenesis inhibitor angiostatin also led to reduced tumor size without changing tumor cell proliferation rates.[44]

The CD36 receptor has been reported to be the receptor on vascular endothelial cells that mediates the antiangiogenic effects of TSP-1.[35] We could not detect differences in endothelial cell CD36 expression between control tumors and TSP-1 overexpressing tumors by immunostaining. However, TSP-1 overexpressing A431 tumors showed increased CD36 expression in tumor cells, as compared to no or little CD36 expression in control tumors. TSP-1 transfected A431 and SCC-13 cell clones also showed highly increased CD36 expression *in vitro*. Moreover, incubation of control A431 clones with conditioned media obtained from TSP-1 overexpressing clones significantly induced CD36 expression. These results suggest that soluble TSP-1 induces the CD36 receptor on tumor cells. They are in accordance with the recent findings that adhesion to TSP-1 induced CD36 in embryonic fibroblasts.[45] Our results further demonstrate that upregulation of CD36 expression in TSP-1 transfected A431 cells moderately enhanced cell adhesion to immobilized TSP-1 *in vitro*, in accordance with a previous report dem-

onstrating that HT-1080 fibrosarcoma cells adhered to TSP-1 via their CD36 receptor. The functional consequences of enhanced tumor cell expression of CD36 *in vivo* remain unknown since we did not detect any differences in tumor cell proliferation between control and TSP-1/CD36 overexpressing A431 xenotransplants. However, it is conceivable that the CD36/TSP-1 system might play a role for the invasive or metastatic properties of malignant tumor cells *in vivo*.

Overexpression of TSP-1 in A431 xenotransplants resulted in extensive areas of tumor cell necrosis, possibly due to anti-angiogenic effects of TSP-1. To define the effects of TSP-1 on tumor vasculature, we performed morphometric analyses on CD31 stained tumor sections by using a semiautomated image analysis program. In TSP-1 overexpressing tumors, a moderate reduction of the microvascular density was observed, as measured by the average number of CD31-positive tumor vessels per mm^2. Similar results have been reported in human breast cancer xenotransplants.[36] However, when we compared vessel sizes, we found that the average vessel area was significantly diminished in TSP-1 overexpressing tumors with absence of larger vessels with surface areas of greater that 2,000 μm^2. These findings demonstrate that TSP-1 reduced the characteristically increased size of tumor vessels and suggest that measuring vascular densities alone may not be sufficient to detect changes in tumor vasculature. They also suggest that determining the total vascular area per unit area may serve as a more sensitive parameter to measure tumor angiogenesis.

It is of interest that the first vascular changes observed during treatment of experimental tumors with an antibody to the angiogenesis factor VEGF also consisted of a dramatic reduction of blood vessel diameters and tortuosity.[46] Moreover, overexpression of VEGF in the skin of transgenic mice[3,3] or in MEL-57 melanoma xenotransplants[4] led to the development of tortuous and dilated blood vessels, and inhibition of the VEGF-inducible $\alpha 1$- and $\alpha 2$-integrins significantly inhibited VEGF-driven tumor angiogenesis *in vivo*, most prominently through reduction of average blood vessel diameters.[47] To exclude that the reduction in vessel sizes observed in TSP-1 overexpressing A431 xenotransplant tumors was due to down-regulation of VEGF expression, we performed *in situ* hybridizations of tumor xenotransplants. These studies demonstrated unchanged levels of VEGF mRNA expression in TSP-1 overexpressing tumors *versus* controls. Therefore, the reduction in vessel sizes reflects an important role of TSP-1 on the formation of tumor vasculature and demonstrates that similar vascular effects can be obtained by overexpression of TSP-1 or by inhibition of VEGF, suggesting antipodal roles of the two molecules in tumor angiogenesis. In summary, TSP-1 induced a potent growth inhibition of malignant epithelial skin cancer with complete inhibition of tumor development of highly differentiated SCC-13 tumors. The anti-tumoral effect of TSP-1 was not due to direct inhibition of tumor cell proliferation, but was associated with significant inhibition of tumor angiogenesis. Our results suggest that quantitation of both microvascular densities and of total vascular areas provides a much more sensitive parameter for tumor angiogenesis than determination of vessel densities alone.

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